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# Use of PNA oligonucleotides for the *in situ* detection of *Escherichia coli* in water

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A species-specific Peptide Nucleic Acid (PNA) oligonucleotide probe directed against the $V_1$ region of the 16S rRNA molecule was synthesized for the detection of *Escherichia coli* in water. The specificity of the probe was tested in dot blot hybridizations against a number of environmental isolates including those from the genera *Escherichia, Klebsiella, Enterobacter* and *Citrobacter. In situ* hybridization experiments were performed with biotinylated PNA oligonucleotide probes with subsequent detection of the biotin label using a combination of Streptavidin-Horseradish Peroxidase and a tyramide signal amplification system. The results obtained enabled the specific detection of *E. coli* in under 3 h.

Hybridizations were also performed on cells which were treated with chlorine (1·5 mg $l^{-1}$) for up to 30 min. *Escherichia coli* cells were still detected after storage for 14 days at room temperature. No cells were detected by conventional plate count or the 'Colilert' assay, a method currently used for the routine detection of *E. coli* and coliforms in the water industry. Cell viability was assessed by the ability of cells to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to highly fluorescent formazan crystals through bacterial respiration. Only cells that had not been chlorinated were detected. These results confirm that ribosomal RNA exists within the cell long after cell death has occurred and that rRNA cannot be used to assess the viability of individual cells. However rRNA probes in combination with viability markers should enable the specific detection of viable cells *in situ*. Hybridization experiments were also performed successfully on seeded tap water samples. The number of fluorescent cells detected correlated well with those obtained by plate count analysis. This represents the first reported use of PNA oligonucleotides for *in situ* detection of micro-organisms and offers a fast efficient alternative to conventional DNA approaches.


***KEYWORDS:*** PNA, *E. coli*, rRNA, viability, probes.

## INTRODUCTION

Peptide nucleic acid (PNA) is a synthetic DNA mimic composed of a peptide backbone based on 2-aminoethylglycine and four nucleobases attached through methylcarbonyl linkages.[1] Although PNA has unique biological, physical and chemical properties it can form strong Watson-Crick complexes with DNA or RNA.[2] Peptide nucleic acid has been used for a wide range of applications including antisense drug discovery, DNA mapping,[3] polymerase chain reaction (PCR) clamping[4,5] and Southern and Northern blotting. However the potential use of PNA oligonucleotides for *in situ* hybridization and detection of micro-organisms has not yet been reported. Hybridization with PNA oligonucleotides to target DNA/RNA offer

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many advantages over their DNA counterparts, they can hybridize rapidly, hybridization is independent of salt concentration,[2] they are resistant to nuclease and protease attack,[6] they bind more specifically and shorter probes can be used for greater sensitivity. The hybridization efficiency of PNA oligonucleotides is significantly greater than their DNA counterparts if the target is embedded in secondary structure.[2] Peptide nucleic acid can also be labelled with reporter molecules e.g. biotin and fluorescein for non-isotopic detection. As PNA oligonucleotides bind strongly to their target site, hybridization times can be reduced to 30 min allowing hybridization and detection to be completed within a few hours.

Identification and detection of coliforms and *Escherichia coli* are of utmost importance to the water industry. At present microbiological water quality is determined using one of a number of methods including membrane filtration, Colilert or most probable number methods. Other alternative methods have also been assessed such as the PCR[7] impedance monitoring and by using enzyme specific substrates[8,9] but each method has produced variable results. Although Colilert is quick and easy to perform (compared to membrane filtration[10]) and can be used by any routine laboratory it requires 18 h for a result to be obtained. There is an increasing need for a rapid test which will detect and correctly identify *E. coli* such that water providers can respond quickly to bacteriological failures of water quality and thereby protect public health.

In recent years molecular methods based on ribosomal RNA targeted probes have been used to identify individual cells in bacterial populations.[11,12] However, there still remains a certain degree of uncertainty as to whether the presence of ribosomal RNA is indicative of cell viability and how long can ribosomal RNA remain within the cell after cell death occurs. Other viability markers such as those based on bacterial membrane integrity, respiration, cell multiplication and nucleic acid stains have been shown to produce variable results,[13,14] therefore there is a need to develop more reliable methods for the differentiation of live and dead cells, especially in the water industry where disinfection is used as the final barrier in water treatment. In the present study we have determined whether the detection of 16S rRNA within the cell, by *in situ* hybridization assays, can be used as an alternative to present viability markers.

The application of *in situ* hybridization techniques for the detection of specific bacteria in environmental samples has been limited. The low ribosome content of cells found in these conditions has resulted in the detection of very low levels of probe-conferred fluorescence. As a result more sensitive and reliable methods are necessary. Alternative methods have been analysed which include the use of *in situ* PCR,[15] *in situ* detection of mRNA[16] and the use of biotinylated and horseradish-radish peroxidase-labelled probes and their subsequent detection with a tyramide signal amplification system[17,18] called TSA (NEN Life Science Products, Boston, MA, USA).

In the present study we have examined the potential use of biotinylated PNA oligonucleotide probes directed against the 16S rRNA molecule for the *in situ* detection of *E. coli* in water. As PNA oligonucleotide probes have been shown to bind to complementary RNA with increased efficiency the use of biotinylated PNA oligonucleotides in combination with the TSA signal amplification system should provide a more rapid and sensitive method for the detection of *E. coli in situ*.

## MATERIALS AND METHODS

### Design of PNA oligonucleotide probes

16S rRNA databases (EMBL and Genbank) were searched for a species-specific signature for the detection of *E. coli*. A species-specific probe (5'GCAAAGCAGCAAGCTC 3') was designed from position 71 to 86 of the *E. coli* 16S rRNA molecule. The PNA oligonucleotide was labelled at the 5' end with biotin and an N-terminal lysine (Oswell, Southampton, UK). The oligonucleotide was dissolved in 10% trifluoroacetic acid and heated to 50°C before use to ensure that the oligonucleotide was completely dissolved.

### Cultures

All strains (Table 1) were grown in nutrient broth at 37°C overnight. Cells were harvested by centrifugation in late exponential phase.

### Dot blot hybridizations

Total chromosomal DNA was isolated by the method of Pitcher *et al.*[19] from a number of environmental isolates (Table 1). Approximately 0·1 μg of DNA was subjected to PCR which involved 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and extension at 72°C for 1 min. The primers used in the reaction were from semi-conserved regions of the 16S rRNA molecule, from position 6-28 and position 1510-1492. The PCR reaction was performed in a total volume of 100 μl

**Table 1.** Results of dot blot hybridizations to amplified rDNA from *Escherichia coli*, other *Enterobacteriaceae* and a number of reference strains

| Organism | Strain | Binding of PNA probe to amplified 16S rDNA |
|---|---|---|
| *Buttiauxella agrestis* | NCTC 12119 | − |
| *Campylobacter coli* | NCTC 11366 | − |
| *Citrobacter fruendii* | *1036 | − |
| *Citrobacter amolonaticus* | *1073 | − |
| *Enterobacter aerogenes* | *890 | − |
| *Enterobacter amnigenes* | *1156 | − |
| *Enterobacter cloacae* | *1001 | − |
| *Enterobacter intermedius* | *884 | − |
| *Enterobacter agglomerans* | *1144 | − |
| *Escherichia coli* | NCTC 9001 | + |
| *Escherichia coli* | *6362 | + |
| *Escherichia coli* | *6852 | + |
| *Escherichia coli* | *6410 | + |
| *Escherichia coli* | *6851 | + |
| *Klebsiella aerogenes* | NCTC 8167 | − |
| *Klebsiella oxytoca* | *1201 | − |
| *Klebsiella pneumoniae* | NCTC 9633 | − |
| *Klebsiella pneumoniae* | *1131 | − |
| *Klebsiella ornitholytica* | *892 | − |

NCTC = National Collection of Type Cultures, Collindale, London, UK.
* Environmental isolates identified by API 20E, +denotes positive reaction, −negative reaction.

containing: 0·5 μg of DNA, 1X Taq polymerase buffer, 2·5 U of Taq polymerase enzyme (Perkin Elmer, Warrington, UK), dNTPs (200 μM of each) and 200 μM of each primer. The amplified products were then denatured by heating to 100°C for 2 min and cooling on ice. The rDNA was then bound to 'Hybond N' membrane (Amersham Life Science, Amersham, UK) using the BioRad (Hemel Hemstead, UK) dot blot apparatus. Membranes were incubated in prehybridization buffer (Sigma-Aldrich company Ltd, Poole, UK) for 30 min at 42°C and then in hybridization buffer (Sigma-Aldrich) with the biotinylated PNA oligonucleotide for 30 min. The membranes were washed and the biotin label detected using the Gene Images CDP-Star detection kit (Amersham Life Science). Membranes were exposed to Hyperfilm™ (Amersham Life Science) for 1 h and developed using the Compact X2 automatic film processor (X-Ograph Ltd, Malmesbury, UK).

### *In situ* hybridization

Overnight cultures were filtered through 0·45 μm 'Costar' metallic membrane filters. The filters were then placed on a membrane filter pad (Whatman, Maidstone, UK) which was presoaked in 6% paraformaldehyde and the cells fixed at room temperature for 20 min. The membranes were rinsed three times in sterile water at room temperature (RT) and the cells lysed by overlaying the filter with 300 μl of lysozyme solution containing 50 μg $ml^{-1}$ of lysozyme followed by incubation at room temperature for 10 min. The membranes were rinsed in sterile water and then hybridized at 50°C for 30 min in 10 mM sodium phosphate buffer pH 7·2 containing 10 μmoles of PNA probe. Following hybridization the membranes were washed at 50°C for 15 min in 500 μl of 20 mM Tris-HCl, 0·01% SDS, 180 mM NaCl, 5 mM EDTA. The membranes were finally rinsed three times in sterile water at room temperature.

### Detection of *in situ* hybridized cells

The PNA biotinylated probe was detected by use of the TSA signal amplification kit (NEN Life Sciences). A modification of the method provided with the TSA kit was used. Briefly membranes were blocked by incubation in 500 μl of 0·1% blocking agent, 0·1% Tris-HCl pH 7·5, 0·15 M NaCl (TNB buffer) at room temperature for 15 min. A 1:100 dilution of streptavidin-HRP in TNB buffer was then added and incubation continued for a further 30 min. The membranes were washed three times for 5 min each in 0·1 M Tris-HCL pH 7·5, 0·15 M NaCl, 0·05% Tween

20 (TNT) buffer. Fluorescein tyramide (300 µl of a 1: 50 dilution in 1X amplification diluent, provided in the TSA kit) was then added and the membranes incubated at room temperature in the dark for 10 min. Membranes were washed three times in TNT buffer at room temperature for 5 min. The membrane filters were then mounted on glass microscope slides in low fluorescence immersion oil (Citifluor UKC, Canterbury, UK), a coverslip placed on top and the membranes viewed under an epifluorescence microscope. The images were captured by use of a cooled charge-coupled device camera (CCD) and analysed with image analysis software (Digital Pixel, Brighton, UK). The images were then saved in TIFF format for importing into Photodeluxe version 2.0 (Adobe, Mountain View, California, USA).

### Detection of *E. coli* cells after chlorination

An 'Instachlor' rapid release chlorine tablet (Wilkinson & Simpson Ltd., Tyne & Wear UK) was added to 50 ml of sterile distilled water. An aliquot (3 ml) of this stock solution was added to each of three vessels containing 100 ml of sterile distilled water. A diethyl-p-phenylene diamine (DPD) tablet was added to 10 ml of solution taken from each of the three vessels. Free chlorine present in the sample reacted with the DPD to produce a pink colouration which was then measured with a photometer at 520 nm according to the manufacturers instructions (ELE International, Hemel Hempstead, Hertfordshire, UK). The chlorine content was adjusted (if necessary) to 1·5 mg $l^{-1}$. *Escherichia coli* cells ($1 \times 10^8$) were then added to the three vessels which were incubated at room temperature for 5, 15 and 30 min, respectively. Free chlorine was then neutralized by the addition of sodium thiosulphate (Fisher Scientific, Loughborough, UK) to a final concentration of 5 µg $ml^{-1}$. The amount of free chlorine present after neutralization was assumed to be zero. An aliquot of cells (100 µl) was filtered through 'Costar' metallic membranes and hybridizations were performed as described above. An equal aliquot of cells were spread onto $R_2A$ agar plates (LabM, Bury, UK) and incubated at 25°C for 5 days. Aliquots (100 µl) were also assessed by Colilert (Idexx, Chalfont St. Peter, UK) according to the manufacturers instructions. Aliquots of cells were also tested after 24 h, 48 h and 2 weeks storage at room temperature. A control sample containing no chlorine was also examined.

### CTC viability assay

Aliquots (100 µl) of 14-day-old samples of non-chlorinated cells and cells that had been treated with 1·5 mg $l^{-1}$ of chlorine for 5, 15 and 30 min were filtered through 0·45 µm black polycarbonate membranes (Whatman). The membranes were then incubated on 25-mm absorbent pads (Whatman) presoaked in 600 µl 40 mM MOPS buffer (Sigma, Poole, UK) pH 6·5, 0·5% glucose containing 4 mM CTC (Polysciences, Warrington, USA) at 30°C for 6 h.[20] Cells were then fixed with 37% formaldehyde and counterstained with DAPI (10 µg $ml^{-1}$, Sigma) at room temperature for 5 min. The membranes were washed in sterile water, air dried and mounted onto glass slides for microscopic observation.

### Detection of *E. coli* in tap water

Samples of tap water (500 ml) were taken from a water distribution system. Free chlorine was neutralized by the addition of sodium thiosulphate. The water samples were then spiked with approximately $10^3$ *E. coli* cells $ml^{-1}$ Samples (10 ml) were then filtered through 'Costar' metallic membrane filters and hybridizations performed with the biotinylated PNA specific probe. Serial dilutions were plated onto $R_2A$ agar plates for accurate cell counts and the plates were incubated at 37°C for up to 3 days.

## RESULTS

### Dot blot hybridizations

A PNA oligonucleotide probe was designed against the 16S rRNA molecule for the specific detection of *E. coli*. The sequence selected was compared to >200 other sequences currently in the EMBL database and found to have two mismatches with its nearest relative. The PNA oligonucleotide was synthesized with an N-terminal lysine and a biotin moiety attached at the 5' end (Oswell, Southampton, UK). The specificity of the PNA oligonucleotide was checked by dot blot hybridizations. Results obtained are shown in Table 1. No cross-reactivity was detected when the probe was tested against other Gram-negative bacteria. All *E. coli* isolates including a number of environmental strains gave a positive signal.

### *In situ* hybridization

*In situ* hybridizations with biotinylated PNA oligonucleotides were performed on 'Costar' metallic membrane filters. Following fixation and permeabilization, hybridizations were carried out at 50°C for 30 min. The biotinylated label was detected

**Fig. 1.** Identification of *Escherichia coli* after hybridization with a species specific PNA probe targeted against the 16S rRNA molecule and subsequent detection with the TSA signal amplification system.

by the addition of streptavidin HRP followed by the catalytic deposition of fluorescein tyramide. The membranes were then viewed under an epi-fluorescence microscope and images were captured by a cool charge couple device camera as shown in Fig. 1. Control experiments were also performed with strains that were negative on dot blot hybridization to ensure that there was no non-specific binding of the probe. Fig. 2 shows the results obtained when the *E. coli* PNA specific probe is used in a mixed population of cells. The results clearly distinguish *E. coli* from other strains examined.

### Detection of *E. coli* cells after chlorination

*In situ* hybridization experiments were also performed on bacteria that had been exposed to disinfection conditions similar to those used in the water industry. *Escherichia coli* cells that had been treated with chlorine (1·5 mg $l^{-1}$) for up to 30 min were still detected immediately after chlorination using the probe system. However, no cells were detected by plate counts or by Colilert on samples that were analysed immediately after chlorination and for up to 2 weeks later. After incubation at room temperature for 2 weeks 10% of the cells were still detected with the species specific probe (Fig. 3, Fig. 4). Cells that were not pre-chlorinated but spiked into water for 2 weeks were still brightly labelled. After 2 weeks aliquots of cells were removed from each treatment and examined for their ability to reduce CTC to insoluble formazan crystals by bacterial respiration. Cells which were not chlorinated were able to reduce CTC to insoluble fluorescent crystals. However no crystals were observed on cells that had been pretreated with chlorine for 5, 15 or 30 min.

**Fig. 2.** Specific detection of *Escherichia coli* in a mixture of environmental isolates containing *E. coli, Klebsiella pneumoniae, Buttiauxella agrestis* and *Enterobacter cloacea*. All cells were counterstained with DAPI. Separate Images were taken of cells stained with DAPI and the *E. coli* specific PNA oligonucleotide probe. The two images were then overlaid to produce a third image, as shown above, which specifically detects *E. coli* (labelled green).

### Detection of *E. coli* in tap water

*Escherichia coli* cells spiked into tap water samples were detected by *in situ* hybridization and plate count assays. The hybridization experiments produced results which were similar to those observed by the plate count method. Figure 5 shows the results obtained when 10 ml of sample was filtered onto 'Costar' metallic membranes and hybridizations performed as described. The cells were clearly visible and no background interference was detected.

## DISCUSSION

Examination of potable water for the presence of coliforms and *E. coli* remains the most frequently performed test for assessing the microbiological quality of water. The presence of these organisms in drinking water is indicative of faecal contamination, biofilm formation or a breakdown in water treatment processes. Present methods used to identify these organisms are often slow and inefficient taking 18 h or more for a positive identification. In the present study we have examined the potential use of *in situ* hybridization experiments in combination with PNA oligonucleotides as a faster and more reliable alternative for the detection of viable *E. coli* in water.

It is currently extremely difficult to distinguish *E. coli* from other closely related taxa using phenotypic traits. In recent years studies have shown that the primary sequences of ribosomal RNA can be utilized to develop highly specific oligonucleotide probes for microbial identification.[11] rDNA probes have been

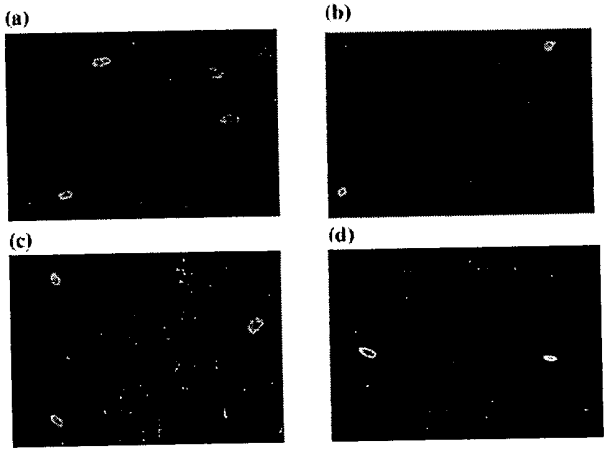


**Fig. 3.** Detection of chlorinated and non-chlorinated cells of *Escherichia coli* after incubation for 14 days at room temperature. (a) *E. coli* non-chlorinated cells; (b) *E. coli* cells pretreated with 1·5 mg $l^{-1}$ of chlorine for 5 min; (c) *E. coli* cells pretreated with 1·5 mg $l^{-1}$ chlorine for 15 min; (d) *E. coli* cells pretreated with 1·5 mg $l^{-1}$ of chlorine for 30 min.

used to successfully identify organisms *in situ*. However, the successful use of these probes for the specific detection of organisms in the environment has been limited. This has been partly due to the low ribosome content of cells, the secondary structure of the rRNA molecule and the low permeability of some cell membranes to large molecules e.g. rDNA probes with biotin or HRP attached.[17,18]

Peptide nucleic acid probes can be used as an alternative to conventional DNA or RNA probes. Hybridization can be performed in solutions that do not contain salt. Under these conditions the highly ordered structure of the rRNA molecule begins to unravel. Fast hybridization times together with the high permeability of these synthetic molecules provide major advantages over their DNA/RNA counterparts.

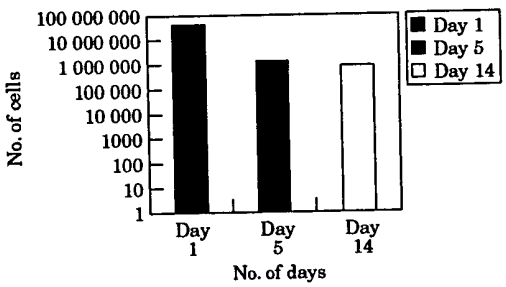


**Fig. 4.** Results of *in situ* hybridization studies on *Escherichia coli* cells that were treated with chlorine and incubated at room temperature for 1, 5 and 14 days. No cells were detected by the plate count and Colilert methods.

In the present study, peptide nucleic acid probes, targeted against the 16S rRNA molecule, were chemically synthesized with a biotin moiety attached at the 5′ end for the specific detection of *E. coli*. Initially the specificity of the PNA probe was checked by dot blot hybridizations against a number of coliform strains found in water. In addition the sequence of the oligonucleotide probe was compared with those presently in the EMBL database. The biotin label

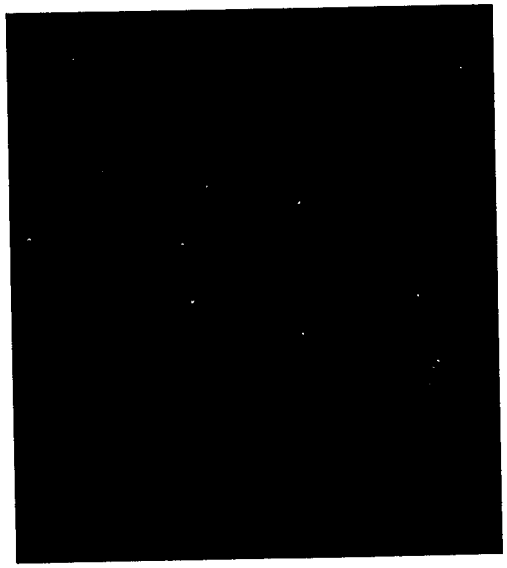

**Fig. 5.** Detection of *Escherichia coli* in tap water using biotinylated PNA specific probes and the tyramide signal amplification system.

attached to the PNA oligonucleotide was detected with streptavidin fluorescein and the CDP star detection module (Amersham Life Science). The results of this study have shown that the 16S rRNA probe is highly specific for *E. coli* with all reference strains examined producing a negative result.

*In situ* hybridization experiments with the biotinylated PNA probe in combination with the Tyramide signal amplification system from NEN Life Sciences were performed on overnight cultures of *E. coli*. Brightly labelled cells were detected in under 3 h. The 30 min hybridization time needed for annealing of the PNA oligonucleotide is much shorter than that used for biotinylated DNA probes providing a significant reduction in the detection time which may be extremely important for industrial processes. The specificity of the hybridization experiments were also assessed *in situ*. A mixed population of cells, containing *E. coli, Klebsiella, Enterobacter* and other *Escherichia* species were also examined. Cells of *E. coli* were clearly distinguishable confirming the ability of the PNA probe to specifically detect the species.

Cells of *E. coli* assessed under stressed conditions also produced positive results. Cells incubated in sterile distilled water for 2 weeks were still detected. Although the number of cells decreased those that were present appeared to have adapted to their nutrient depleted environment. These cells were able to reduce the redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride), through bacterial respiration, to insoluble intracellular formazan crystals which are highly fluorescent. The cells did not require any pre-enrichment step to maximize their rRNA content. This is particularly important as selection for a small proportion of the total cell number in an environmental ecosystem should not occur. This may be due to the ability of the PNA oligonucleotides to bind more efficiently to their target sites especially if embedded in secondary structure or their ability to bind to a higher proportion of rRNA molecules within the cell. Hybridizations were also performed with conventional DNA probes (data not shown). However there was a wide variation in the probe-conferred fluorescence obtained and a large number of cells produced very weak signals (data not shown).

*Escherichia coli* cells that were chlorinated and examined by *in situ* analysis also produced positive results. The signal obtained was comparable to that of non-chlorinated cells. Initially this may not be surprising because although rRNA synthesis would have ceased rRNA would presumably exist within the cell for a period of time after cell death. As no cells were detected on $R_2A$ agar plates or with the commercially available 'Colilert' system these cells were assumed to be dead. After 48 h incubation at room temperature there was a significant drop in the population detected by rRNA *in situ* hybridizations. However, although the level of fluorescence and therefore the rRNA content of those cells detected decreased significantly after 48 h, cells were still detected after 2 weeks incubation in water at room temperature. As a result aliquots of cells were taken and examined for their ability to reduce CTC. No respiratory activity was detected on cells that were chlorinated for 5, 15 or 30 min. This result agreed with those obtained with the plate count and 'Colilert' system.

The ability of the PNA probes to detect *E. coli* accurately in tap water samples offers the potential to develop these methods for routine analysis. However the results presented have confirmed that ribosomal RNA probes cannot be used as an indication of the viability of an organism after chlorination. Ribosomal RNA appears to survive for relatively long periods of time after cell death has occurred. This may be because the highly ordered structure of the molecule makes it resistant to chemical attack. Although ribosomal RNA can still be used to specifically identify an organism it may be necessary to use other viability markers when deciding on whether a cell is live or dead. However the ability of the PNA oligonucleotides to hybridize rapidly to their target offers a fast alternative to conventional cultural and biochemical techniques. As *E. coli* remains an important indicator organism for the microbiological quality of drinking water the ability to identify it in under 3 h (compared to 18 h) would enable fast response times to be achieved if contamination of the water supply were to occur. Clearly further work is required to establish if the procedure could be adopted routinely, but the findings presented here clearly demonstrate the possibility of using such a procedure in emergencies.

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